Morphological Features of the Initial Portion of Intralingual Lymphatic Vessels in the Sprague–Dawley Rat Tongue: An Enzyme–Histochemical and Electron Microscopic Observation

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Abstract **Objective** To elucidate the initial portion of intralingual lymphatic vessels in the tongue of rat. **Methods** The morphological features of the initial portion of intralingual lymphatic vessels and their spatial relations to the connective tissue cores were examined histochemically by a combined light and electron microscopy. The differentiation of initial lymphatics from blood capillaries was performed by a 5′−nucleotidase(5′−Nase) staining method. On the tissue surface, 5′−Nase−positive lymphatic capillary walls were seen as highlights by backscattered electron imaging−scanning electron microscope (BSI−SEM). **Results** Various initial lymphatics were seen in all four types (filiform, fungiform, foliate and vallate) of lingual papillae on tongue dorsal surface. A simple or twisted lymphatic loop accompanying with blood capillaries was as possible as extent to intrapapilla and the lymphatics were shorter in the prominence. The radiated lymphatics with blood capillaries in the fungiform papilla showed a basket or petal−like network. The lymphatic and blood capillary structures in ring−like network surrounding the epithelial sulcus can be observed in the foliate papilla. A conglomerated loose network of the lymphatics and blood capillaries projected into second papilla (taste buds) in vallate papilla. The initial lymphatics form horizontal network in the lamina propria. **Conclusion** The morphology and distribution of initial lymphatics may be corresponded to the shape of the connective tissue core and the surface structure.

Key words Rat lingual papilla; initial lymphatics; 5′−nucleotidase; enzyme−histochemistry

The lymphatic drainage of the tongue is of clinically importance for the understanding of the lymphatic metastasis of oral and neck tumor, especially when we need further study on the details of morphological feature of the initial portion of the intralingual lymphatic, with special reference to the close relation of the absorption of tissue fluids and various free cells. In the present study, we try to elucidate in the initial portion of the intralingual lymphatic vessels in the tongue of the rat with combined 5′−nucleotidase (5′−Nase)−alkaline phosphatase (ALPase) double staining and electron microscopy. In the whole region of subepithelial tissue layer, 5′−Nase−positive lymphatics were observed in the intralingual connective tissue papillae, the lamina propria mucosa of the tongue, being associated with ALPase−positive blood capillaries. The number of lymphatics network in the tip and body of tongue increased obviously compared with that in the root of tongue. Moreover, their morphological features differ from the four type papillae of the tongue. In spite of this numerous lymphatic without valve−like structures, coursing vertically, were seen in the lamina propria mucosa of the tongue. In general, lingual lymphatics have many communicating branches between the connective tissue papillae and the lamina propria. We think most of all initial lymphatics were originated from blind−endings at the apical parts of intralingual papillae and form horizontal network in the lamina propria mucosa of the tongue and the enlarged in muscle layer.

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Morphological studies of the lymph drainage in the tongue have been predominantly carried out by traditional injection techniques, which demonstrated the lymphatic gross contour and lymph flow of the tongue [1,2], the fine structure feature of lymphatic endothelium has been enriched by the findings of transmission electron microscopy[3~6]; Furthermore, the surface morphology of the initial lymphatics have been demonstrated in the rat tongue by scanning electron microscopy [7~9]; But these studies had shown that rich lymphatic plexus occurs only at the muscle and lamine propria layer from where lymphatic ramified seldom extends into intrapapillae. Many of these studies were focused on the initial lymphatic distribution in the lamina propria of the tongue. As a means of elucidating special pathological processes in tongue cancer [10], little information is available on lymphatic vessels in the lamina propria extend to papillae. The papillae serve as chemo−and mechano−receptor, which is one of the most sensitive indicators of many diseases. Thus, it would be of interest and importance to identify the lymphatic fine structure in the papillae and may be helpful in understanding the physiology of the lingual mucosa. To the best of our knowledge, there have been no studies on the distribution and architecture of initial lymphatic in the four type papillae of the tongue. Many investigation have examined the three−dimensional lymphatic in the lingual[11, 12], however information on the relation between the lymphatics of connective tissue papillae and surface structure are not yet clear. Recent reports show that 5′−neucleotidase (5′−Nase) as an important enzyme in nucleotide metabolism, is being widely employed not only to differentiate between blood capillary and lymphatic capillary [13]. The three dimensional features and fine structure support a concept for the enzyme−histochemical differentiation of a small lymphatic from the blood capillary [14]. Investigation with 5′−nucleotidase (5′−Nase) and alkaline phosphatase (ALPase) enzyme have been proved as a reliable clue for studying intraorganic lymphatics and blood capillaries [15~17], and which have been widely applied to various organs.

In this experiment, the enzyme −histochemical method was applied to the research on lymphatics. In view of uncertainties or lacking of information on the extent, pattern and distribution of initial lymphatic capillaries in the tongue, we chose the four types papillae of the tongue because of its specific structure and functional importance. In order to subject of this study, the 5′−Nase and ALPase staining method were used to identify the lymphatic capillaries and blood capillaries, and to clarity the three dimensional lymphatic architecture of lingual papillae with spatial relations to the connective tissue papillae and surface structure.

MATERIALS AND METHODS

Experimental animals and tissue preparations

Fourty adult Sprague −Dawley rats of both sexes, weighing 180g to 350g, were examined. All of the experiments were carried out according to the Laboratory Animal Committee of the Harbin medical University. All rats were anesthetized with intraperiotoneal pentobarbital (40mg/kg). Tissue for enzyme−histochemistry examination were fixed by perfusion via the left ventricle with 100ml of physiological saline followed by 100ml of 4%~6% paraformaldehyde −1% $CaCl_2$ 0.1M cacodylate buffer (pH=7.2) containing 7% sucrose. After perfusion, the tongue were excised. For whole−mount preparation of intact or peeled tissue carefully wipe out of muscle with optic scissors and then stretched out over the steels spines on a plastic plate with the papillae side facing up. The specimens were immersed in 0.1M cacodylate buffer (7% sucrose) and were fixed the above−mentioned fixative solution for 1 hour or 2 hour at 4 ℃.

Most of the material was taken from the posterior area of dorsum of the tongue near the orophargnx. Pieces of the tongue from the following areas, ① vallate papilla; ②foliate papillae; ③ an area of epithelium from the dorsal surface of the anterior part of the tongue which contained both filiform papillae and scattered fungiform papillae; ④ an area of epithelium from the posterior surface containing conical papillae, embedded in frozen in OCT compound at a temperature of 34℃, were removed. Frozen sections with 10μm~15μm were cut with cryostat (Leica CM1100) at a cabinet temperature 18℃ for OCT blocks and mounted on APS coated slide and dried at room temperature.

Reagents

(1) 5′−neucleotidase Solution [0.2M Tris−meleate buffer (pH=7.2) 20ml; Adenosine 5′−monophosphate sodium salt 25mg; 0.1M $MgSO_4$ 5ml; Sucrose 3~4g; Distilled water 22ml; 2%Pb $(NO_3)_2$ 1.5ml]; (2) Alkaline phosphate solution [Nophthol AS−MX phosphate disodium salt 40mg; N, N−dimethyformamide 2ml; 0.1M Tris−HCl buffer (pH=9.2) 40ml; Fast blue BB or Variamine blue RT 40mg].

Tissue preparation

After immersing in 3.5N sodium bromide solution over night at 4℃, the epithelial layer was carefully pealed off from the intrinsic lingual muscle layer with the manual method. For whole−mount preparation, the subepithelial tissue layer was stretched, then pinned flat on a plastic plate and mounted with the epithelial sides facing up, and immersed in 2% paraformaldehyde fixative in 0.1M cacodylate buffer (pH=7.2) containing 10% sucrose for 1 hour at 4℃.

Enzyme−histochemistry

For the light microscopy, whole−mount preparations

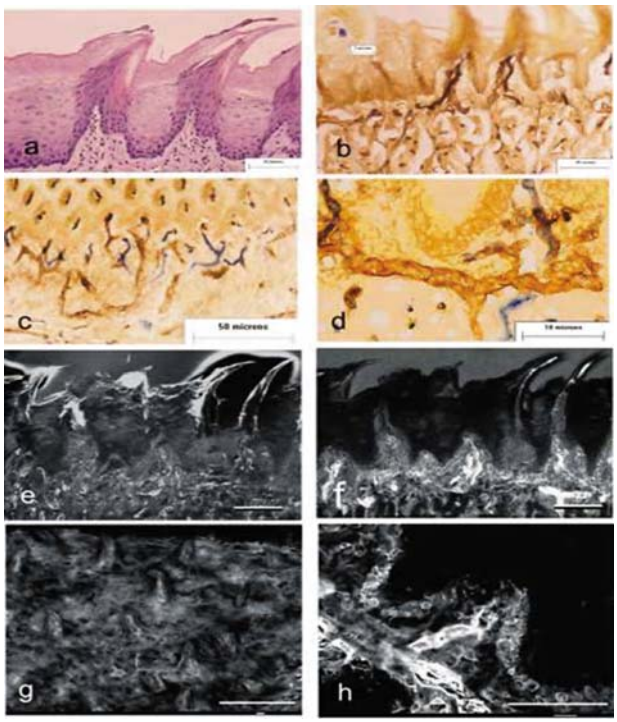

Fig.1 Light micrograph of sagittal section of the filiform papillae in apex of tongue of the rat.
a: HE staining Bar: 20μm
b,c and d: Lymphatic capillaries are stained in black−brown color and blood vessels are stained in blue color in the connective tissue and muscular layer . (5′−Nase−ALPase double staining)
b:Bar: 20μm, c: Bar: 50μm , d: Bar: 10μm
e: SEM view Bar: 100μm
f: SEM view of backscattered imagings in the same section as e after staining with 5′−Nase. Lymphatic networks were strongly highlighted. Bar: 100μm
g: BEI−SEM view of whole−mount preparatation lymphatics in each filiform papilla was highlighted and lymphaticks distributed in the lamina propria mucosa are found. Bar: 100μm
h: BEI−SEM view of frontal cryostat section Bar: 50μm

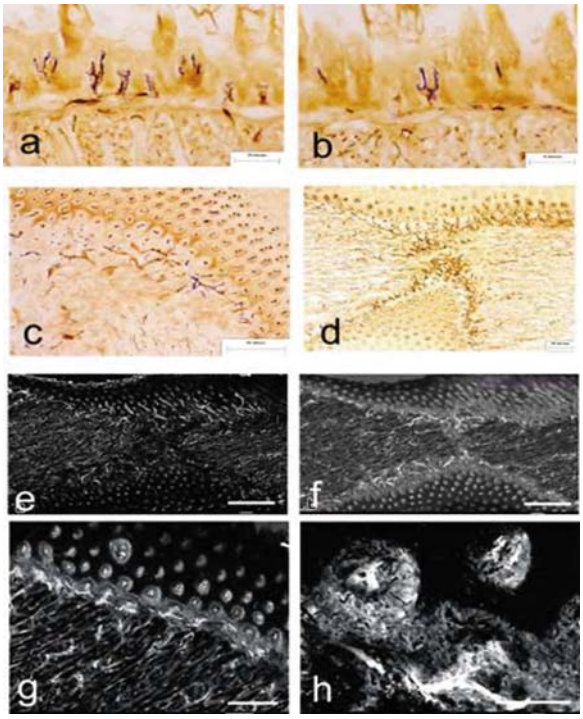

Fig.2 The view of the filiform papillae in the prominence of the dorsal mucosa of the rat tongue.
a and b: Frontal section showing the lymphatics (brown)were shorter than blood capillary (blue). 5′−Nase−ALPase double staining. Bar: 20μm
c and d: Horizontal section 5′−Nase−ALPase double staining. Bar: 50μm
e: SEM view of horizontal cryostat section the blood capillaries may be highlighted. Bar: 50μm
f: SEM view of BEI in the same section as e after staining with 5′−Nase the lymphatics were strongly highlighted. Bar: 50μm
g and h: BEI view of frontal section.
g: Bar: 202μm, h: Bar: 50μm

and OCT frozen tissue sections were processed with 5′−Nase lead−based medium (35 min, 37℃) for lymphatics and ALPase azo−dye reaction medium for blood capillaries. They were observed under a light microscope.

Scanning electron microscopy

After staining for 5′−Nase activity of lymphatics, several whole−mount preparation were subjected to backscattered electron imaging−scanning electron microscopy at 15 or 20 Kv(S−3400N,BSI−SEM).

Transmission electron microscopy

The materials were fixed in PFA fixation for 2 hours. Small tissue blocks of various regions were reacted with 5′−Nase[18] for 30 min at 37℃ and then post-fixed in 2% osmium tetroxide solution at 4℃ for 2 hours. This was followed by dehydrated in a graded series of enthanol and embedded in epoxy resin. Ultrathin sectioning and U−Pb double staining. The specimens were observed under transmission electron microscope (JEOL JEM 1220 EX).

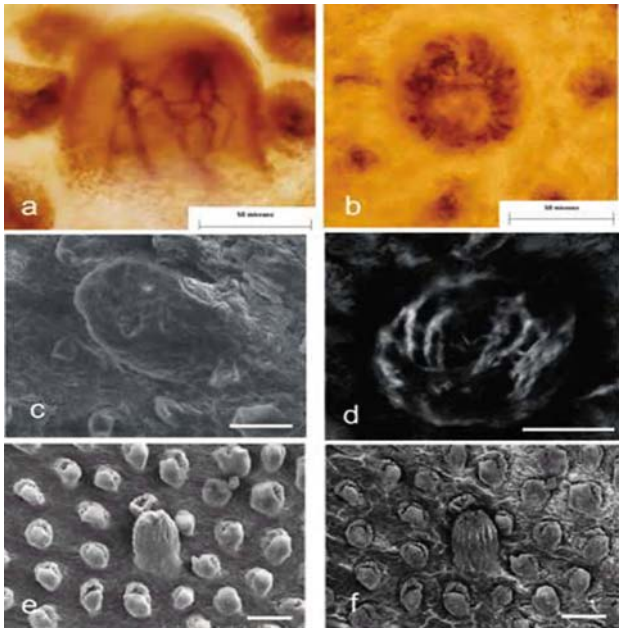

Fig. 3 The view of whole−mount preparatation in fugiform papillae of the rat.

a: Blood capillaries revealed a basket or petal−like network ALPase staining Bar: 50μm

b: The radiate shaped lymphatics. 5′−Nase staining Bar: 50μm

c: SEM view Bar: 50μm

d: The radiate shaped lymphatics were highlighted.BEI−SEM. Bar: 50μm

e: SEM view Bar: 100μm

f: SEM view of backscattered imagings in the same section as e after staining with 5′−Nase. Lymphatic networks were strongly highlighted. Bar: 100μm

RESULTS

The structural organization of the lingual papillae of rat was observed by scanning electron microscopy method described by Iino T: ① filiform papillae in a narrow sense were distributed at the anterior part of the tongue. Connective tissue of those papillae was of roughly conical shape. ② Connective tissue papillae of each fungiform had a sea anemone−like structure at the top where there was taste bud. ③At the anterior margin of the intermolar prominence, there were large conical papillae of the connective tissue cores. ④ in the posterior part of the intermolar prominence, there were branched papillae which had connective tissue papillae with several small protrusions. ⑤The connective tissue of the foliate papillae appeared as several elliptical holes

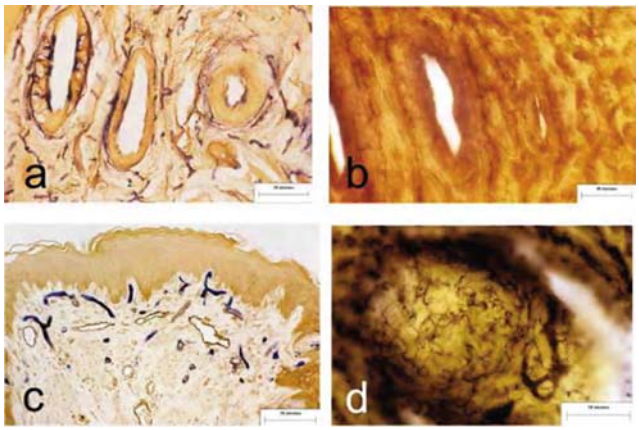

Fig.4 The view of the foliate and vallate papilla of the dorsal mucosa of the rat tongue.

a: Horizontal section of a foliate papilla of the rat, Dark brown lymphatic networks (arrows) are distributed around the foliate papillae 5′−Nase−ALPase double staining. Bar: 20μm

b: whole−mount preparation of a area of foliate papillae. Bar: 40μm

c: Frontal section of vallate papilla of the rat showing lymphatic capillary (brown) and blood capillary (blue), (taste buds) 5′−Nase−ALPase double staining. Bar: 20μm

d: whole−mount preparation of a area of vallate papillae showing a conglomerated loose network of the lymphatics projected into second papilla (taste buds) in vallate papilla. for 5′−Nase staining. Bar: 50μm

at each groove whose internal edge was raised upwards.

We observed the lymphatic and capillary architecture underlying the lingual papillae of the rat and compared the structure which is different in the location of each type papillae. The fine structure of initial lymphatic vessels (ILV) and their distribution in the normal rat tongue were examined. We found that the morphological features of the initial lymphatic vessels vary greatly in form in the CTC of the tongue and the structure of the initial lymphatic capillaries is different in four types of papillae, and lymphatics spring from the extensive lamine propria network.

In routine histological preparation, lymphatic vessels (Fig.1a, 3c) cannot always be recognized because they are often collapsed and difficult to be distinguished from connective tissue space. However, if specimens are processed using 5′−Nase staining by electron backscattered imaging microscopy, lymphatic will be highlighted; 7~10μm thickness OCT embedded sections are examined using 5′−nucleotidase (5′−Nase) and Alkaline phosphatase (ALPase) double staining with the light microscope, the lymphatic capillaries were stained in black−

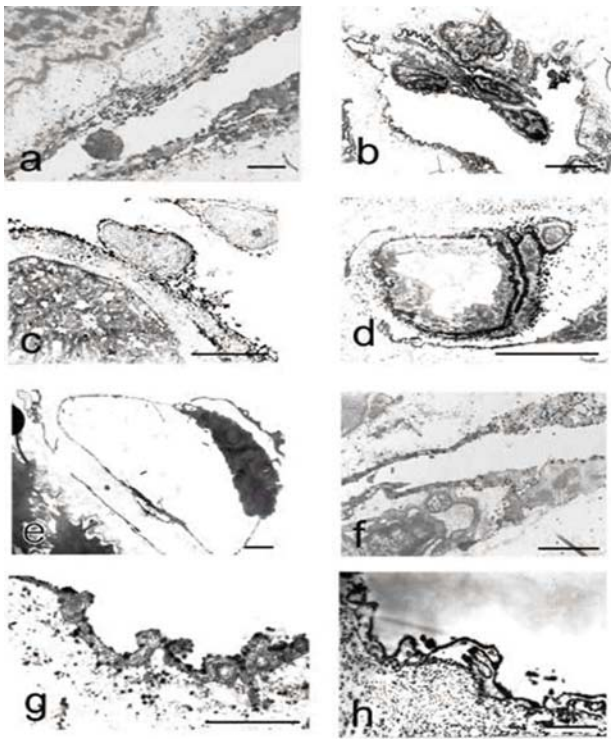

Fig.5 TEM view of 5′−Nase−positive lymphatics in the intralingual papilla.

a: The reaction products for 5′−Nase activity are indicated on the endothelial cell surface.

Bar: 1μm

b and c: Lymphatic endothelial cell surrounded by 5′−Nase reaction granules. Bar: 5μm

d: Most of the lymphatic capillaries in rat tongue have no active function with imperforation or collapsed cavity in normal physical state. Bar: 5μm

e: Fenestrated capillary in the intralingual papilla Bar: 1μm

f: Typical intercellular overlapping junctios of Note 5′−Nase reaction product extending into the junctional interface of the endothelial cells. Bar: 1μm

g: end−to−end junction Bar: 1μm

h: interdigitating junction Bar: 1μm

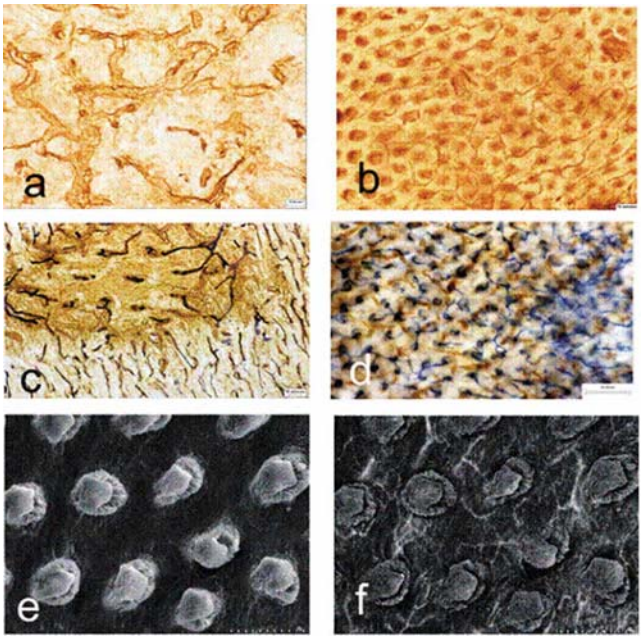

Fig.6 The view of the lamina propria layer of the dorsal mucosa of the rat tongue.

a: Horizontal section showing brown colored extensive lymphatics network. 5′−Nase staining Bar: 10μm

b: whole−mount preparation showing brown the blind−endings of lymphatics from intralingual papilla into lamina propria layer form lymphatic network. 5′−Nase staining. Bar: 10μm

c: Horizontal section showing brown colored extensive lymphatics network accompany with blue colored blood capillaries network. (5′−Nase−ALPase double staining) Bar: 10μm

d: whole−mount preparation showing brown the blind−endings of lymphatics from intralingual papilla into lamina propria layer form lymphatic network. (5′−Nase−ALPase double staining)

Bar: 50μm

e: SEM view Bar: 100μm

f: BEI−SEM view of whole−mount preparatation lymphatics in each filiform papilla was highlighted and lymphaticks distributed in the lamina propria mucosa are found.

Bar: 100μm

brown, the blood capillaries were stained in blue-violet color. Fine distributed of 5′-Nase-positive initial lymphatic and ALPase-positive blood capillaries intralingual papillae.

Filifom papillae

(1) in the filiform papillae: the 5′-Nase positive lympatics capillaries showed a simple or twisted lymphatic loop accompanied by blood capillaries, which entered into the secondary papillae(Fig.1 b, c, d); 5′-Nase-positive lymphatics were highlighted in BEI-SEM images (Fig.1 e, f, g, h). (2) Large conical shape filiform papillae: The lymphatics of 5′-Nase positive were observed in cryostat section with the possession of three humps, which entered into the connective tissue cores, the lymphatics humps are relatively shorter than the blood capillaries (Fig.2 a-h).

Fungiform papillae

The abundant lymphatics are widely distributed in the intralingual. In the whole-mount preparations, the radiated-shaped initial lymphatics in the fungiform papillae around furthermore (Fig.3 b, d), the radiate-shaped of 5′-Nase-positive lymphatics were also observed highlighted in fungiform papillae on SEM backscattered images of the tissue blocks (Fig.3 f). The blood capillaries form a basket or peal-like network in the central intralingual papillae (Fig.3 a), their tips was complicated.

Foliate papillae

Foliate papillae with a coin-like appearance are present along the side of the tongue, which are composed of about 4 to 5 ridges. The brown lymphatic and blue blood capillaries form flat ring-like network surrounding the epithelium sulcus (Fig.4 a, b).

Vallate papilla

There is one vallate papilla which is surrounded by a deep circular. Many small tufts of hairin-shaped brown lymphatic were observed. There were almost not extend to the secondary papillae, however, the conglomerated blood capillaries loops were arranged on the superficial portion of papilla in the whole-mount preparation and the blood capillaries were observed to extend into secondary papillae (Fig.4 c, d).

Transmission electron microscopy

In the TEM, the cerium-based 5′-Nase activity staining method could be detected the reaction product on the surface of the lymphatic endothelial cells (Fig.5 a, e, f). No detectable 5′-Nase activity was observed in the blood capillaries (Fig.5 h). Lymphatic capillaries are different in intralingual and propria layer of the rat tongue. Most of the intralingual lymphatic capillaries in rat tongue have no active function with imperforation or collapsed cavity in normal physical state (Fig.5 g). In propria layer, the relationships between adjacent endothelial cells are variable, consisting of end-to-end adhesion (Fig.5 c) with desmosome-like junctions, overlapping cytoplasmic processes (Fig.5 b) or fork-like interlocking (Fig.5 d). There are few open junctions in the normal rat tongue.

In the rat tongue, the initial lymphatic vessels shows its own special shape in the four types intralingual papillae, whose branches extend to lamina propria layer form the thicker lymphatic network totally (Fig.6 a~f).

DISCUSSION

The lymphatic system is very similar to the blood vascular system from venous capillaries onwards. There are innumerable small, thin-walled vessels which merge centrally into large one progressively. Generally, the initial lymphatic are usually some 100~500μm long and of vary irregular shapes. They have ′maximal diameters ′of 15~75μm when completely filled[19], but they are usually quite flattened. They are normally arranged in complex, approximately two dimensional networks [20]. The enzyme-histochemical staining techniques involving 5′-Nase and ALPase has widely been used in studying the distribution and fine structural of lymphatics and blood vessels in various organs of several mammalian [21,22]. The surface morphology of the initial lymphatics has been demonstrated in the rat tongue by scanning electron microscopy [7, 23, 24]. Ohtani have observed that many of the initial lymphatics in rat cecal mucosa start with blind-ends by chemical digestion [25]. Furthermore, Kato et al.

revealed the overall structural organization of initial lymphatics and capillaries of the mesentery by histochemical method [26].

The overall structural organization of initial lymphatics capillaries and collecting lymphatics of the intralingual papillae revealed by our histochemical method is schematically presented in Figure 7. The apical parts of the 5′−Nase−positive initial lymphatics arise with knob−like blind ending. The differentiation of initial lymphatics from blood capillaries was performed by a 5′−nucleotidase (5′−Nase) staining and BSI−SEM method. Consequently, the histochemical method seems very important in locating the lymphatics. Under light and electron microscopy there are numerous blood capillaries and lymphatic vessels at the interface between the intra papillae to lamina propria. The little projections of the mucous papillae called the connective tissue core, which contain a supply of both blood capillaries and lymphatic vessels. Moreover, certain diseases, for example, lymphatic metastasis of the cancer in oral and neck regions may cause main alteration of the intralingual lymphatics which are of importance in diagnosis and treatment. The fundamental to the whole system is the function of the initial lymphatics, which remove the lymph from the tissue.

Ultrastructural studies on the lymphatic vessels [19, 27] have provided a more adequate description for the lymterns and their surrounding tissue (the dimensional lymphatic structure of the lingual papillae remained to be fully elucidated due to the limited to light microscopy). Subsequently, lymphatics of the vesical wall were depicted by light and electron microscopy. However, to clarify initial lymphatic capillary distribution, we now examined the fine structure of small lymphatic vessels (SLV) and their distribution in the normal rat tongue. Particular attention directed to the structure of the lymphatic capillaries in different types papillae were still not in detail. In the present study, we have clearly observed 5′−Nase positive initial lymphatic vessels in sections and whole mount preparation of lingual papillae and confirmed histochemically the initial lymphatics distribute are different in respective type intrapapilla. The results indicate that 5′−Nase−positive lymphatics begin blind sac formation within intralingual papilla.

The cerium−based reaction produced a granular electron−dense precipitate on the cell surface of lymphatic endothelium and gave better ultrastructural localization for 5′−Nase activity. Lymph formation depends only on the drainage of small amounts of lymph scattered through these blind−ends of intrapapilla lymphatics within the connective tissue core (CTC). The disagreement among previous studies, including whether intralingual papilla lymphatic vessels exist, could be due to the uncertainty in detecting lymphatic capillaries without special staining, and may also be related to interspecies differences and the criteria employed for dividing intralingual papilla and lamina propria areas. The initial lymphatic is of considerable interest that no open junctions of the lymphatic endothelial cells are evident in the intralingual, most of the initial lymphatic capillaries in the rat tongue have no active function with imperforation or collapsed cavity in normal physical state. In this respect, the lymphatics may be similar to those of the kidney, thyroid gland and pancreas; but lamina propria lymphatics are similar to the dermis [15, 28] and diaphragm[29] in which typical lymphatic intercellular overlapping, end−to−end and interdigitating junctions are a notable characteristic and constitute a major thorough fare for transport [30]. Initial lymphatics play important roles not only in interface between the intra papillae to lamina propria, but in the regulation of lymph drainage and the pathogenesis of certain diseases.

REFERENCES

1. Katayama K. Lymphatic system of the oral cavity. Kaibogaku

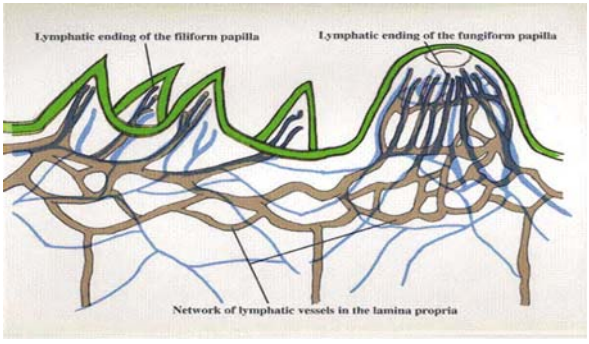

Fig.7 A schematica presention of the initial lymphatic and capillary in the rat tongue intralingual papillae according to the enzyme−histochemical study.

journal Acta Anatomica Nipponica, 1936, 10: 3.

2. Miura K. comparative anatomical study the lymph system in the oral cavity. Kumamoto medical Journal (Japan), 1961, 35: 15−28.

3. Casley Smith JR, Dc.S, BM.The fine structure and functioning of tissue channels and lymphatics. Lymphology, 1980,12: 177−18.

4. Albertine, K.H., O'Morchoe C.C.C: Renal lymphatic ultrastructure and trans−lymphatic transport. Microvasc. Res, 1980, 338−351.

5. Courtice, F.C.,The lymphatic circulation. In: Schwartz,C.J., Werthessen,N.T. and Wolf, S. Structure and function of the circulation, Plenum Press. New York, 1980, 2: 487−602.

6. Leak, L.V. Lymphatic vessels. In: Johannessen, J.V. Electron microscopy in human medicine. McGraw −Hill, New York, 1980, 5: 155−212.

7. Castenholz A. Morphological characteristics of initial lymphatics in the tongue as shown by scanning electron microscopy. Scanning electron microscopy, 1984, 3: 1343−1352.

8.Castenholz A. Interpretation of structural patterns appearing on corrosion casts of small blood and initial lymphatic vessels. Scanning electron, 1989, 3: 315−325.

9.Castenholz A. Functional microanatomy of initial lymphatics with special consideration of the extracellular matrix. Lymphology, 1998, 31: 101−118.

10. Endo M. An experimental study on the distribution and structure of lymphatic capillaries in the connective tissue underlying induced tongue cancer. Dent J Iwate Med Univ, 1993.18: 36−50.

11. Fujimura A, Segawa K, Aita N, *et al.* A morphological study of the capillary structures underlying the lingual dorsal mucosa of the golden hamster. Jpn J Oral Biol, 1988, 30: 75−82.

12. Fujimura A, S.S., Liao MY, Hu X, Onodera M, Nozaka Y., Three dimensional architecture of lymphatic vessels in the tongue. Lymphology, 2003, 36: 120−127.

13. Werner JA, Schunke M, Tillmann B. Histochemical visualization of lymphatic capillaries in the rat: A comparison of methods demonstrated at the posterior pharyngeal surface. Arch histol Jpn, 1987, 50: 505−514.

14. Kato S. Enzyme −histochemical identification of lymphatic vessels. Jpn J Lymphology, 1989a, 12: 13−24.

15. Ji RC, Kato S. Enzyme −histochemical study on postnatal development of rat stomach lymphatic vessels. Microvasc Res, 1997, 54: 1−12.

16.JI RC. Enzyme −histochemical investigations on the lymphatics of the intrinsic uterine wall in the monkey. Biomed Res ,1998, 19 : 347−355.

17.Ji RC, Kato S. Intrinsic Interrelation of lymphatic endothelia with never elements in the monkey urinary bladder. Anat Rec, 2000, 259: 86−96.

18. Kato S, Miyauchi R. Enzyme−histochemical visualization of lymphatic capillaries in the mouse tongue: Light and electron microscopic study. Okajima Folia Anat Jpn, 1989b, 65: 391−404.

19. Casley−smith J.R., Florey H.W. The structure of normal small lymphatic. Quart J.Exp. Physiol, 1961, 46: 101−106.

20. Fujimura A, Seki S, Liao MY, *et al.* Three dimensional architecture of lymphatic vessels in the tongue. Lymphology, 2003, 36:120−127.

21. Koto S, Miura M. Enzyme−histochemical staining method for lymphatics and blood capillaries −A Re−examination of 5′−nucleotidase −alkaline phosphatase double staining with special reference to variation in different tissue and species. Jpn J Lymphology, 1993, 16: 9−17.

22.Kato S, Miura M. A differential staining method for blood vessels and lymphatics in tissue section of some animals and human. Jpn J Lymphology, 1994,17:1−6.

23. Castenholz A. The endothelium of initial lymphatics during postnatal development of the rat tongue. Scanning electron Microsc, III: 1985, 1201−1208.

24. Castenholz A. Structureal and functional properties of initial lymphatics in rat tongue: Scanning electron microscopic findings. Lymphology, 1987, 20: 112−125.

25. Ohani, O. Structure of lymphatic of in rat cecum with special reference to submucosal collecting lymphatics endowed with smooth muscle cells and valves. Scanning electron microscopic study. Arch Histol Cytol, 1992, 55: 429−436.

26.Kato S, Miura M, Nakamura E. Enzyme −histochemical demonstration of lymphatic vessels in the subserosal layer of organs by tissue spread method. Jpn J Lymphology, 1993, 16: 19−26.

27. Leak, L.V. Electron microscopicobservation on lymphatic capillaries and the structural componets of the connective tissue-lymph interface. Microvasc Res , 1970, 2: 361−391.

28. Leak, L.V., Burke, J.F. Ultrastructural studies on the lymphatic anchoring filaments. J cell Biol, 1968, 36: 129−149.

29. Casley−smith, J.R., Lymph and lymphatics. In: Haley, G. and Burton M.k., Microcirculation. University Park Press, Baltimore, London/Tokyo, 1977, 1: 403−502.

30. O′Morchoe C.C.C.. Lymphatic system of the pancreas. Microsc Res Techn, 1997, 37: 456−477.